Conditional p53 Deletion Promotes Adult Neurogenesis and Improves the Acquisition and Clearance of Contextual Fear Memory

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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2014

Abstract

We crossed Nestin-CreER\textsuperscript{T2} mice and p53 flox mice to produce mice with heterozygous or homozygous p53 deletion in a tamoxifen (TAM)-dependent manner (named iKO-p53\textsuperscript{+/-} and iKO-p53\textsuperscript{-/-} mice, respectively). Result showed that dividing Ki-67\textsuperscript{+} cells and immature DCX\textsuperscript{+} neurons in the dentate gyrus were increased in iKO-p53\textsuperscript{+/-} and iKO-p53\textsuperscript{-/-} mice 4 weeks after the TAM injection. To study the retrograde effect of p53 deletion on behaviors, mice received TAM after the training in the CFC and was tested 4 week later. iKO-p53\textsuperscript{+/-} mice displayed degraded contextual fear memory. To study the anterograde effect of p53 deletion on behaviors, mice received TAM 8 weeks before the training in the CFC and was tested o1 day after the training. Results showed a trend of increased contextual fear memory. Therefore the result provide evidence that increased adult neurogenesis is associated with improved acquisition and clearance of hippocampus-dependent contextual fear memory.
Acknowledgments

I thank Dr. Yosuke Niibori, Russell Smith and Antonietta DeCristofaro for their technical support. I also thank Dr. Adelaide Yiu, Frances Xia and Axel Guskjolen for editing of the thesis. Finally, I thank Dr. Paul Frankland for his supervision, guidance and steady support of this work.
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Introduction

1.1 History of studying adult neurogenesis

Neurogenesis, a process of new functional neurons being generated from progenitor cells and incorporated into neuronal circuits, had long been believed to occur exclusively during prenatal and early perinatal development (Ramon y Cajal 1913). The “no new neurons after birth” dogma was first challenged by Altman and colleagues in 1965. They used tritiated thymidine ([3H]-TdR) to label and identify newborn neurons based on morphology providing the first anatomical evidence of continuous neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG) (Altman and Das 1965) and in the subventricular zone (SVZ) (Altman et al. 1969) in adult rats. This was confirmed by a study combining electron microscopy imaging and [3H]-TdR labeling showing ultrastructural characteristics of new neurons, such as dendrites and synapses (Kaplan and Hinds 1977). Later, functional integration of new neurons was first observed in songbirds (Paton and Nottebohm, 1984) in which neurons responding to sound were also [3H]-TdR labeled. Anatomical integration (Stanfield and Trice 1988, Cameron et al. 1993, Markakis and Gage 1999) and functional integration (van Praag et al. 2002) was later observed in rodents.

Studies of adult neurogenesis were greatly advanced with the introduction of bromodeoxyuridine (BrdU) (a thymidine analog to mark DNA replication) (Kuhn et al. 1996, Kempermann et al. 1997), and retrovirus-based techniques (van Praag et al. 2002) as lineage tracers. Life-long continuous neurogenesis has been found in most mammals studied including human (Eriksson et al. 1998). Recently, CreER\textsuperscript{T2} based transgenic models were developed (Lagace et al. 2007, Ninkovic et al. 2007; Imayoshi et al. 2008), providing even better spatial and temporal specificity for birth-dating and manipulating newborn neurons and neural progenitors compared to previous methods. Since then a number of molecules, such as Notch-1 (Ables et al. 2010), fragile X mental retardation protein (FMRP) (Guo et al. 2011) and ERK5 (Pan et al. 2012), have been identified to regulate adult neurogenesis and also hippocampus-dependent learning behaviors.

Although rapid progress has been made to decipher the functions and regulation of adult neurogenesis owing to the advanced techniques, a number of significant questions remain, such as the origin of adult born neurons, factors regulating the adult neurogenesis and their functional
significance to suggest a few. Building on previous studies, the present is aimed to investigate the impact of increased adult neurogenesis on hippocampus-dependent behavior.

### 1.2 The hippocampal circuitry

SGZ adult neurogenesis is not only a series of developmental events in adult brains. To completely understand its functional significance and how it is regulated by neural activity, it is necessary to take the surrounding hippocampal circuitry into account. The classic hippocampal circuitry is the tri-synaptic loop (Fig. 1), in which information flows from the layer II neurons of entorhinal cortex (EC) to the dentate gyrus (DG) through the perforant pathway. Dentate granular cells (DGCs) relay the information to CA3 through mossy fibers. CA3 pyramidal neurons send projections to the CA1 through Schaffer collaterals and CA1 pyramidal neurons project back to the deep layer of EC completing the loop. Additionally, CA3 neurons receive direct input from layer II neurons of EC through the perforant pathway and CA1 neurons receive direct input from layer III neurons of EC through the temporoammonic pathway. This simplified model depicts only major unidirectional excitatory pathways which are incomplete as DGCs receive complex excitatory and inhibitory input.

First, DGCs receive additional excitatory input from CA3 and ipsi- and contralateral hilar mossy cells shown by a study using a dual-viral monosynaptic retrograde tracer (Vivar et al. 2012). In particular, immature DGCs at 21-30 day post injection (dpi) receive transient direct excitatory input from mature DGCs and relatively strong CA3 back projection implying that these projections may function to facilitate the integration and survival of immature DGCs.

Second, there are complex feedforward and feedback inhibitory circuits in the DG consisting of multiple types of interneurons (Fig. 2). The interneurons in the DG are categorized as six types (three categories) based on the location of their cell bodies and distribution of their axon terminals (reviewed in Houser 2007).
The first category of interneurons has their cell bodies located at the hilus where they receive excitatory input from DGCs and provide feedback inhibition. Hilar perforant path associated (HIPP) cells project to the outer two thirds of the molecular layer where DGCs connect to the perforant path from the EC. Similarly, hilar commissural-association pathway-related (HICAP) cells target the inner one third of the molecular layer where DGCs receive projection from hilar mossy cells which provide the first glutamatergic input to the adult-born DGCs (Chancey et al. 2014).

The second category of interneurons has their cell bodies located at the molecular layer where they receive excitatory input from the EC and hilar mossy cells, and thus serve feedforward
inhibition. Between them, the molecular layer perforant path-associated (MOPP) cells target the outer two thirds of the molecular layer and the molecular layer granular cell layer-associated (MOGCL) cells target granular cell layer (GCL).

The third are Parvalbumin-positive basket cells and axoaxonic cells with their cell bodies located at SGZ and targeting GCL. The former targets soma or perisomatic dendrites of DGCs and the latter targets axon initial segments. They both exert strong feedback inhibition on DGCs.

![Figure 2. It shows the cell bodies and axon targeting regions of 5 types of interneurons in the DG (Wojtowicz 2012)](image)

Finally, DGCs are innervated by cholinergic septal cells. Specifically, immature DGCs at 21 dpi receive relatively strong cholinergic input (Vivar et al. 2012). Previous studies have shown a positive correlation between cholinergic activation and adult neurogenesis. Injecting neurotoxin to the cholinergic forebrain resulted in decreased neurogenesis in the DG (Cooper-Kuhn et al. 2004), while activation of cholinergic signaling with donepezil, an acetylcholinesterase inhibitor, increased cell survival in the DG (Kaneko et al. 2006). However, the function of cholinergic input onto mature DGCs is unknown.
1.3 Adult neurogenesis in the DG

1.3.1 Stages in differentiation

It is now widely accepted that continuous adult neurogenesis occurs in two neurogenic loci in mammals: the SGZ of the DG, where new granule cells are generated and added to the hippocampal circuits (reviewed in Gage 2000); and the SVZ of the lateral ventricles, where differentiation and development of new neurons occurs during the migration along the rostral migratory stream (RMS) to the olfactory bulb to become GABAergic interneurons and glutamatergic juxtaglomerular neurons (Lois et al. 1996, Brill et al. 2009, reviewed in Ming and Song 2011). The magnitude of adult neurogenesis varies from species to species, such that unlike rodents, humans generated very limited (if any) new olfactory bulb neurons (Bergmann et al. 2012).

Adult hippocampal neurogenesis is a continuous process (Fig. 3) which initiates from Nestin-expressing Type I cells located in the SGZ (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). Type I cells and astrocytes share several markers such as; glial fibrillary acidic protein (GFAP), Sry-related HMG box transcription factor (Sox2), brain lipid binding protein (BLBP) and glutamate-aspartate transporter (GLAST). Type I neurons can be differentiated from astrocytes by its expression of Nestin, a marker specific for early neural progenitors, and by their characteristic radial processes extending through the granular cell layer into the molecular layer (reviewed in Kempermann et al. 2004, Hsieh 2012). Some studies dub Type I cells as neural stem cells (NSC) based on in vitro evidence showing their quiescence and self-renewal properties and multi-pluripotent differentiation into neurons and glia (Palmer et al. 1995, 1999; Reynolds and Weiss, 1992). However, in vivo studies often show conflicting results regarding whether Type I cells do in fact differentiated into astrocytes (Bonaguidi et al. 2011, Dranovsky et al. 2011, Encinas et al. 2011, Lagace et al. 2007).

Type I cells respond tonically to ambient GABA which maintains their quiescent state. Once committed to asymmetric division, Type I cells enter a series of transient amplifying progenitor (TAP) stages (Type IIa, Type IIb cells) and neuroblast stage (Type III cells). In this progression, Type I cells gradually lose their stem cell features as they acquire neuronal features. Specifically, Type IIa cells have short processes, express Nestin and Sox-2 but not GFAP, and are able to differentiate into neurons or astrocytes in vivo (Suh et al. 2007). Contrary, Type IIb cells are
Nestin positive but Sox-2 negative, and they start to express an early neuronal marker Doublecortin (DCX) (Fukuda et al. 2003; Suh et al. 2007). Type III cells express DCX but not Nestin and have no processes (Dhaliwal and Lagace 2011). Finally, when they exit the cell cycle, they become DCX, Calretinin and NeuN-expressing immature DGCs. Next, the excessive immature DGCs generated compete for BDNF to survive (Sairanen et al. 2005). The survivors integrate into the local network at 1 to 4 weeks of age (Ge et al. 2006). At 8 weeks, newborn DGCs become physiologically indistinguishable to developmental-born DGCs (van Praag et al. 2002, Zhao et al. 2006, Stone et al. 2011).

Although much is now known at the various stages of newborn DGC development, there remains unknowns about progenitors, such as whether there are common progenitors for DGCs and glia cells under physiological conditions (Bonaguidi et al. 2011), how and when the fate-decision is made, and how much heterogeneity there is with neural progenitors and stem cells (DeCarolis et al. 2013).

Figure 3. Markers expressed by developing cells at different stages (Modified from Hsieh 2012).
1.3.2 Integration and maturation of immature DGCs

The integration and maturation of immature DGCs can be divided into two periods. Anatomical integration happens most extensively during the first period, and functional integration during the second. In both periods, immature DGCs exhibit hyper-plasticity compared to mature DGCs.

The first critical window is from 1 to 4 weeks post injection (wpi) (determined by BrdU) when immature DGCs compete for survival and integration. By retrovirus-based birth-dating and lineage tracing method, Zhao and colleagues showed that as early as 3 dpi, immature DGCs begun to extend dendrites to the molecular layers and axons to the hilus and CA3 (Zhao et al. 2006). As in embryonic and neonatal neurogenesis, immature DGCs express high levels of NKCCI (importing [Na$^+$], [K$^+$] with 2 [Cl$^-$]) channels which sets the resting membrane potential more negative than the reversal potential of [Cl$^-$]. They are therefore tonically depolarized by ambient GABA released by neighboring interneurons which regulates spine formation (Ge et al. 2006). The majority of inhibitory synapses are formed between 7-14 dpi with input from the hilus (Ge et al. 2006, Zhao et al. 2006). From 14-21 dpi GABAergic input switches from excitatory to inhibitory in the immature DGCs as they switch from NKCC1 expression to KCC2 (exporting [K$^+$] and [Cl$^-$]) expression (Ge et al. 2006). Next, the majority of excitatory synapses receiving input from the EC and hilar mossy cells are formed between 14-28 dpi (Ge et al. 2006, Kim et al. 2012, Chancey et al. 2014). From 21-30 dpi, immature DGCs receive transient excitatory input from mature neurons and strong innervation from septal cholinergic cells (whose functions are still unclear) (Vivar et al. 2012). Meanwhile, at 16 dpi and onward, axons of newborn DGCs reach CA3 neurons and output synapses start to form (Zhao et al. 2006, Toni et al. 2008). Even most of the efferent and afferent connections are formed before 4 wpi, 4-week old DGCs just started to be functionally integrated as they express low level of IEGs following kainic acid (KA) or Pentylenetetrazol (PTZ) induced seizures (Jessberger and Kempermann 2003). At this stage, newborn DGCs have a lower threshold for LTP induction, smaller LTP amplitude, higher input resistance and lower membrane capacitance compared to mature DGCs (Ge et al. 2007; Ye et al. 2005).

The second period is from 4 to 7 weeks, when spines and synapses continue to grow on newborn DGCs before they become fully mature and functionally integrated into hippocampal network. Immature DGCs begin to robustly express IEGs (immediate early genes), such as c-fos, zif268
and Homer1A following kainic acid (KA) or Pentylenetetrazol (PTZ) induced seizures, contextual fear learning and spatial learning (Jessberger and Kempermann 2003; Stone et al. 2011; Kee et al. 2007). Kee et al. (2007) studied the expression of IEGs in cells of different ages following spatial learning and testing in MWM and found that there were maximal percentage of BrdU+ cells expressing c-fos when the mice were trained in MWM at 6 weeks after BrdU (Kee et al. 2007). Newborn DGCs at this stage exhibit larger LTP amplitude and lower threshold for LTP induction for at least two mechanisms (Schmidt-Hieber et al. 2004; Ge et al. 2007, 2008). On one hand, high level of NR2B expression is believed to play an instructive role in this phenomenon as LTP was more reduced with ifenprodil (NR2B subtype specific antagonist) than with APV (generic NMDAR antagonist) in case that these two drug were administrated to decrease similar level of excitatory postsynaptic potential (EPSP) (Ge et al. 2007). On the other hand, immature DGCs are less sensitive to inhibitory input as stimulation of perforant pathway induced similar level of LTP in immature DGCs with or without bicuculline (an antagonist of GABA\(_A\) receptors) while the same stimulation elicited LTP in mature DGCs only in the presence of bicuculline (Ge et al. 2007, 2008). This is likely due to the late formation of perisomatic GABAergic input onto developing DGCs such that no feedforward inhibition was observed in 4-week old DGCs which had already established most of their excitatory and inhibitory connections (Espósito et al. 2005). These data collectively show that immature DGCs at 4-7 weeks are more functionally integrated into the local network compared to younger DGCs. More importantly, because of their higher excitability and plasticity, they are more responsive to the input compared to mature DGCs (Kee et al. 2007). Related to their characteristic properties, immature DGCs are found to play a central role in certain cognitive functions such as pattern separation (Nakashiba et al. 2012, Kheirbek et al. 2012, Sahay et al. 2011), which will further be discussed in more details.

1.3.3 The life and death of newborn neurons

As in developmental neurogenesis, excessive adult-born neural progenitors and DGCs are generated in the SGZ. Competition for survival occurs at both the progenitor and early postmitotic stages. In regards to the critical window of survival there are differences between mice and rats which should be specified because most of studies about adult neurogenesis were done in these two species. First, mice have shorter adult hippocampal neurogenesis cell cycles. In a study examining BrdU labeled cells in mice at multiple time points after a single injection of
BrdU, the cell cycle length was estimated to be 14 h and the length of the S-phase (phase targeted by BrdU), was 6 - 7.6 h in mice (Mandyam et al. 2007). This time frame fits the finding in another study using BrdU and [3H]-TdR injections at various time intervals from which the mouse cell cycle is estimated to be 12 – 14 h (Hayes and Nowakowski 2002). In a similar study design using BrdU/[3H]-TdR double labeling (Cameron and McKay 2001) in rats, the estimated cell cycle is 24.7 h and the length of S-phase 9.5 h. The difference in cell cycle and S-phase length in mice and rats suggests that the probability of labeling dividing cells is different in these 2 rodent models. In rats, there are 38% of dividing cells at S-phase (Cameron and Mckay 2001), and 42-54 % in mice (Hayes and Nowakowski 2002, Mandyam et al. 2007).

Second, it has been suggested that in rats the adult-born DGCs mature faster than those in mice. In a study directly comparing adult neurogenesis in these two species (Snyder et al. 2009), both mice and rats received one injection of 200 mg/kg BrdU, a dose shown sufficient to label most dividing cells at S-phase in both species (Cameron and McKay, 2001; Mandyam et al., 2007). At 1 wpi, both species showed similar proportions of BrdU/DCX+ double-labeled cells out of total BrdU labeled cells. But from 1 to 4 wpi the proportion of BrdU/DCX cells dropped more and proportion of BrdU/NeuN cells increased more quickly in rats compared to mice. However, it is not clear whether adult neurogenesis in rats is faster also at the progenitor stages.

Third, programmed cell death (PCD) occurs at different time points during adult neurogenesis in rats and mice (Fig. 4). In rats, newborn cells labeled with two injections of 200 mg/kg BrdU or one injection of 5.0 Ci/kg [3H]-TdR reach a maximum at 7 dpi and decrease during 1 to 4 wpi concomitant to the integration of newborn DGCs (Cameron et al. 1993; McDonald and Wojtowicz 2005; Snyder et al. 2009). Cameron et al. 1993 noted a sharp decrease of [3H]-TdR labeled cells between 1 to 2 wpi which are not seen in later studies with BrdU. This may be due to technical inaccuracy because [3H]-TdR labeled cells can be detected only a few microns deep (Sidman et al. 1959; Bisconte et al. 1979) and sections of 50μm thickness were used in the Cameron et al.1993 study.

In mice the peak of BrdU labelled cells is at 1 d after a single injection of 150 mg/kg BrdU (Mandyam et al. 2007), at 36 h post injection after 4 injections of 50 mg/kg BrdU within 12 h and at 48 h post injection after 8 injections of 50 mg/kg BrdU within 24 h (Hayes and Nowakowski 2002). At 1 wpi, the number of BrdU labelled cells decrease at least 50% compared
with the peak, and the highest rate of loss of BrdU labelled cells occurs between 2-4 dpi suggesting different kinetics of newborn cells in mice and rats (Mandyam et al. 2007). In mice but not in rats, the majority of newborn cells commit PCD before the stage of immature neurons. This result is confirmed by a study monitoring microglia-mediated removal of decaying DGCs (Sierra et al. 2010).

One explanation for the difference in BrdU labeling kinetics in rats and mice is their cell cycle length. The mouse cell cycle is shorter, therefore the BrDU dose would dilute faster with each cell cycle and would thus require a higher BrdU dose than rats. However, studies cited typically use the same dose for mice and rats. Additionally, studies conducted to determine saturation doses examined BrdU-labeled cells shortly after injection not taking into account a potential dilution effect. Therefore, the BrdU-labeled cells peak at 7 dpi in rats may be too long for BrdU-labeled cells detection in mice if the BrdU dose is not adjusted for their shorter cell cycle.

The other possibility is that since the rat cell cycle is slower, and newborn cell development is faster (in terms of the time when majority of cells enter postmitotic stage and start to compete for integration) at the postmitotic stage, it’s possible that the number of newborn progenitors cells has not yet reached the threshold for massive selection for survival to occur before exiting cell cycle compared to mice.

In conclusion, the majority of neural progenitors and immature neurons commit PCD before 1 wpi, a time when immature DGCs start to integrate into the local network in mice. During the postmitotic stage, another 25% of immature neurons (compared to the maximum) are eliminated and the rest are integrated into the local network and reach full maturation (Mandyam et al. 2007; Sierra et al. 2010).
Figure 4. Different dynamics of BrdU-labelled newborn cells in mice and rats. A-B. Dynamic of newborn cells in mouse DG with a peak at 1 dpi following a single injection of 150 mg/kg BrdU. B shows the first 60 hours after BrdU injection corresponding to the dashed window in A. (Mandyam et al. 2007); C. Dynamic of newborn cells in juvenile (5-6 weeks old, black bars) and middle-aged rats (12 months old, gray bars) DG with a peak at 7 dpi following two injections of 200 mg/kg BrdU (McDonald and Wojtowicz 2005).

1.4 Regulation of adult neurogenesis

1.4.1 Regulation by growth factor

The adult neurogenesis in SGZ is regulated by several growth factors including the neurotrophin brain-derived neurotrophic factor (BDNF), neural growth factor (NGF) and vascular endothelial growth factor (VEGF) (Frielingsdorf et al. 2007; Jin et al. 2002; Zhao et al. 2007). Among them, BDNF is a major positive regulator of adult neurogenesis.
First, BDNF level was found to be correlated to the hippocampal adult neurogenesis in various physiological and pathological conditions that influence the level of adult neurogenesis. For instance, voluntary running (Russo-Neustadt et al. 2004), environmental enrichment (Rossi et al. 2006), dietary restriction (Lee et al. 2000) and chronic antidepressants (Malberg et al. 2000; Russo-Neustadt et al. 2004; Xu et al. 2003) promote the hippocampal adult neurogenesis and increase the expression of BDNF at the same time. Conversely, physiological and pathological conditions correlating with reduced adult neurogenesis also exhibit reduced level of BDNF, such as stress (Smith et al. 1995; Rasmussen et al. 2002) and Huntington’s disease (Lazic et al. 2004, Zuccato et al. 2001). Importantly, functions of BDNF are causally linked to the change of neurogenesis in these conditions. For example, environmental enrichment-induced neurogenesis was blocked in mice with BDNF heterozygous deletion (Rossi et al. 2006). Antidepressants failed to induce hippocampal adult neurogenesis in BDNF heterozygous mice or mice with impaired trkB function (Li et al. 2008, Sairanen et al. 2005) (trkB is the corresponding receptor for BDNF). Furthermore, infusion of BDNF has antidepressant-like effects (Shirayama et al. 2002) and induces hippocampal adult neurogenesis (Pencea et al. 2001).

However the role of BDNF in adult neurogenesis is complex. Mice with BDNF conditional knockout in mature neurons exhibit increased proliferation (Chan et al., 2008). BDNF heterozygous mice show increased cell proliferation and decreased cell survival when sacrificed 48 h or 21 d after BrdU labeling, respectively. Chronic antidepressants induce cell proliferation in BDNF heterozygous mice sacrificed 48 h after BrdU labeling, but these cells do not survive after 21 days (Sairanen et al. 2005). Another study with deletion of dendritic form of BDNF in mice also found increased cell proliferation and largely decreased cell survival (Waterhouse et al. 2012).

In summary, BDNF is a key factor in the regulation of adult neurogenesis. It also underlies many physiological and pathological conditions regulating adult neurogenesis. Although complex, the main effects of BDNF seem to be negatively regulating cell proliferation but positively enhancing survival of new-born cells.

NGF and VEGF were also found to increase adult neurogenesis in rodent SGZ. NGF promotes survival but not proliferation of newborn neurons. Frielingsdorf et al. (2007) suggested that the effect is at least in part mediated by increased activity of cholinergic septal cells which was
previously found to be positively correlated with the survival of newborn neurons (Cooper-Kuhn et al. 2004; Kaneko et al. 2006). VEGF promotes the proliferation of NSCs and progenitors indirectly through increasing angiogenesis (Mustonen and Alitalo 1995) and directly through interaction with VEGFR2/Flk-1 receptors on newborn cells (Jin et al. 2002; Schänzer et al. 2004).

1.4.2 Regulation by neural activities

The adult neurogenesis in SGZ takes place in a region with complex neural network. The proliferation and survival of new-born DGCs are found to be regulated by past experience, for example by environmental enrichment (Bruel-Jungerman et al. 2005; Kempermann et al. 1997). Moreover, certain hippocampal-dependent learning tasks that induce LTP (Martin and Morris 2002; Lynch et al. 2004) can increase adult neurogenesis specifically in DG (Gould et al. 1999; Snyder et al. 2005), suggesting that adult neurogenesis is under the regulation of local neural activities. Both excitatory glutamatergic and inhibitory GABAergic transmission have composite effects on SGZ neurogenesis.

Local excitation was first found to negatively regulate SGZ neurogenesis. Exogenous NMDA application decreased proliferation of neural progenitors, while lesion of neurons in entorhinal cortex, the main excitatory source to the DG, increased cell proliferation in the DG (Cameron et al. 1995). Systemic injection of AMPA or NMDA receptors antagonist also increased new-born cells in the DG (Bernabeu and Sharp 2000).

However, activation of NMDA receptors has also been suggested to have a direct pro-neurogenesis effect on NSC and progenitors. First of all, NR1 and NR2B subunits of NMDARs are expressed in GFAP+ type 1 NSCs implying a direct effect from excitatory input (Nácher et al. 2007). In both hippocampus-derived neurosphere and cultured hippocampal neural progenitors, new-born neurons were increased by glutamate or NMDA via promoting neuronal differentiation (Deisseroth et al. 2004; Kitayama et al. 2004). In vivo inhibition or activation of calcium ion channels respectively decreased or increased neuronal differentiation in DG (Deisseroth et al. 2004). In vivo artificially induced LTP also promote the proliferation and survival of new-born DGCs (Bruel-Jungerman et al. 2006).
The two conflicting effects of local excitatory activity on adult neurogenesis may be in part reconciled by two recent studies. In Tashiro et al. 2006, researchers used retrovirus-based method to selectively delete NR1 subunit in proliferating cells in the DG. They found that NMDA antagonist increased the number of new-born neurons in NR1 conditional knockout mice, but not in wild-type (WT) animals, suggesting that the anti-neurogenesis effect of local excitation does not function though developing SGZ cells but rather through other types of cells. This notion was further supported by a study examining the effect of interneurons activation on NSCs (Song et al. 2012). The authors found that photoactivation of channelrhodopsin-2-expressing PV+ interneurons increased the proportion of quiescent NSC. Conversely, social isolation increased the proportion of dividing NSCs. Deletion of γ2 subunit of GABA receptor blocked these effects, suggesting that the regulation on NSC activity from PV+ interneurons is exerted through GABA-γ2 signaling. Therefore the direct effect of excitatory input on developing SGZ cells is to promote their survival and proliferation. Simultaneously local excitation may inhibit adult neurogenesis through local inhibitory network.

Additionally, GABA-signaling has direct pro-neurogenesis effect on neural progenitors and immature neurons in the DG. In these cell types, resting potential is lower than the reversal potential of [Cl-] because of the expression of Nkcc1. Therefore the net effect of GABA activation is depolarizing, through efflux of [Cl-], and influx of [Ca2+] by activation of voltage-gated calcium channels (Deisseroth et al., 2004). Tonic GABA activation in Type-2 neural progenitors elevates the expression of NeuroD, a transcription factor that positively regulates neuronal differentiation, and later the number of mature Calbindin+ neurons (Tozuka et al. 2005). At early postmitotic stage, tonic GABA activation positively regulates the integration and survival of immature DGCs (Ge et al. 2006). GABA-mediated neuronal differentiation are proposed to function through calcium influx, as calcium influx mediated by activation of NMDA receptor also induces NeuroD expression and neuronal differentiation, while blocking voltage-gated calcium channel with nifedipine, has opposite effect (Deisseroth et al. 2004).

To summarize, adult neurogenesis is regulated by local neural activity. The excitatory and inhibitory activity both exert multi-faceted functions. Whether the effect is pro- or anti-neurogenesis depends on the stages of neuron development and whether it is mediated directly by developing cells or indirectly through other cells.
1.5 Adult neurogenesis in behavior

1.5.1 Adult neurogenesis and depression

The correlation between adult hippocampal neurogenesis and depression is built on two main observations. First, stress, a well-accepted cause of depression, negatively regulates cell proliferation in the DG (Gould et al. 1992; Gould et al. 1998). Second, many treatments alleviating depression (e.g., voluntary running, environmental enrichment, electroconvulsive therapy, and anti-depressants) increase adult hippocampal neurogenesis (Scott et al. 2000; van Praag et al. 1999a; Malberg et al. 2000; Kempermann et al. 1997). Critically, adult hippocampal neurogenesis is necessary in mediating antidepressant-like effects. Many antidepressants fail to produce behavioral effect on irradiated mice, although decreased adult neurogenesis by x-irradiation itself did not induce depression (Santarelli et al. 2003, Airan et al. 2007, Li et al. 2012). Mice with adult neurogenesis decreased by transgenic or radiation methods exhibited slower recovery from chronic stress (Snyder et al. 2011). Conversely, a recent study demonstrated that increasing adult neurogenesis by tamoxifen-induced bax conditional knockout did not have antidepressant-like effects on its own but augmented the antidepressant-like effects produced by voluntary running (Sahay et al. 2011). In another study, the effect of antidepressants were potentiated by tamoxifen-induced Neurofibromin conditional knockout which increased adult neurogenesis but had no antidepressant-like effects (Li et al. 2012).

Collectively, these studies suggest a role of adult hippocampal neurogenesis in facilitating antidepressant-like effects, but hippocampal neurogenesis on its own does not seem to directly regulate mood or correlate with depression.

1.5.2 Adult neurogenesis and hippocampus-dependent learning behaviors

The functional significance of adult neurogenesis in hippocampus-dependent learning and memory were first suggested by the finding that adult neurogenesis in the DG is regulated by both environmental and physiological factors associated with hippocampal functions. For example, housing in an enriched environment which increased the long term survival of new-
born DGCs improved spatial learning in Morris water maze (MWM) (Kempermann et al. 1997; Nilsson et al. 1999) and both increased memory in contextual fear conditioning (CFC) and facilitated hippocampal LTP (Duffy et al., 2001). Similarly, voluntary running which increased both cell proliferation and long term survival of new-born DGCs in mice improved performance in MWM (van Praag et al. 1999a, 2005). Finally, hippocampus dependent learning, such as trace eyeblink conditioning and Morris water maze, but not hippocampus-independent learning, increases the survival of 1-week old DGCs in rats (Gould et al. 1999; Epp et al. 2007).

Further, the importance of adult neurogenesis in hippocampus-dependent learning tasks is ascertained with various functional knock-out experiments. Shors and coworkers found diminishing adult neurogenesis by administrating methylazoxymethanol acetate (MAM) impaired performance in trace eyeblink conditioning (a hippocampus-dependent associative learning task) but not in the hippocampus-independent delay eyeblink conditioning task (Shors et al. 2001, 2002). Focal x-irradiation can also ablate adult neurogenesis in the DG and impair hippocampus-dependent learning, for example in both place-recognition (Madsen et al. 2003) and CFC (Saxe et al. 2006, Winocur et al. 2006). Similar deficits are found in mice with adult neurogenesis ablated pharmacologically with temozolomide (TMZ) (Garthe et al. 2009) and in transgenic mouse models (Imayoshi et al. 2008, Saxe et al. 2006).

However, conflicting results regarding the functional significance of adult neurogenesis abound. For example, in contrast to van Praag et al. 1999a and Clark et al. 2008, Wojtowicz and coworkers found that increasing hippocampal neurogenesis via voluntary running did not enhance performance in the Morris water maze in rat and only marginally improved contextual fear memory (Wojtowicz et al. 2008). Deficits in contextual fear memory after irradiation is found in some studies (Saxe et al. 2006; Winocur et al. 2006) but not others (Clark et al. 2008). Irradiation usually does not impair the formation of spatial memory in MWM in rat or mouse (Wojtowicz 2008; Clark 2008; Meshi 2006; Saxe et al. 2006; Snyder 2005), whereas deficits in recent memory in MWM was shown when adult neurogenesis was reduced by MAM or by Ganciclovir (GCV) in Nestin-tk transgenic mice (Deng 2009; Goodman 2010).

There are several likely candidates underlying these discrepancies. First, the involvement of adult-born DGCs is higher in rat than in mouse. Snyder and coworkers labelled dividing cells in rats and mice with same dose of BrdU. At 1wpi, the number of BrdU labelled cells normalized to
the volume of GCL was comparable in both species. However at 4 wpi there were more BrdU labelled cells in rats indicating higher survival rate (Snyder et al. 2009). Therefore at any given time point there should be more immature DGCs presented in the hippocampal circuitry in rat. This finding explains the fact that rats are more sensitive to manipulations reducing adult neurogenesis. For example, in Snyder et al. (2009) same dose of irradiation impaired recent contextual fear memory in rats but not in mice. Similar discrepancy can also be found comparing Kitamura et al. (2009) and Snyder et al. (2005). Conversely rats are less sensitive to manipulations increasing adult neurogenesis (see Wojtowicz et al. 2008, van Praag et al. 1999a and Clark et al. 2008). Following this logic, the age of animals at the beginning of experiments also affect the baseline of adult neurogenesis and the result of behavior tests. Reducing adult neurogenesis with TMZ led to deficit of spatial learning in 1-mouth old mice but not in 2-mouth old mouse (Martinez-Canabal et al. 2013).

Second, many of these manipulations are not specific to neurogenesis or neurogenic regions. High dose (>15 Gy) of irradiation often result in necrosis, demyelination and vascular pathology (Calvo et al. 1988; Hodges et al. 1998). Even though the dose used in the studies of neurogenesis is usually lower (<10 Gy) and less devastating, it still causes chronic inflammation in the DG (Monje et al. 2002). Although most experiments using irradiation as the mean to decrease adult neurogenesis conducted behavior experiments at least 2 month afterwards when the pro-inflammatory cytokines subsided to baseline (Clark et al. 2008, Saxe et al. 2006. Snyder et al. 2005), it is still impossible to rule out the chronic effect of irradiation-induced inflammation. Moreover, following acute and chronic low dose irradiation, phosphorylation of PKB (Protein Kinase B), ERK 1/2 (Extracellular signal-regulated kinases) and CREB (cAMP response element-binding protein) is increased in the frontal cortex of male mice (Silasi et al. 2004). The increased CREB activity in the frontal cortex may indicate compensating mechanisms following irradiation, which may help to explain the lack of spatial learning deficit in irradiated rodents (Wojtowicz 2008; Clark 2008; Meshi 2006; Saxe et al. 2006; Snyder 2005).

Running induces BDNF expression in mature DGCs and in other brain regions, such as caudal neocortex (Neeper et al. 1995), and promote brain-wide protein synthesis in a non-specific manner (Nadel et al. 2013). Similarly environmental enrichment induces BDNF in various cortical regions (Ickes et al. 2000). This might help to explain the fact that the facilitation of learning seen in early studies using running or environmental enrichment (Kempermann et al.
1997; van Praag et al. 2005) has not manifested in later studies using more specific transgenic model (Sahay et al. 2011).

Third, neurons of different ages have different characteristics in terms of their electrophysiological properties and connectivity with surrounding circuitry. For example, 4 weeks old newborn DGCs are found to be preferentially involved in hippocampal memory formation (Kee et al. 2007; Ramirez-Amaya et al. 2006). As such, the specific age of the population of adult generated DGCs targeted critically affects experimental outcomes. For example, x-irradiation often induces transient inflammation that can last for about two months (Monje et al. 2002; Meshi et al. 2006). Typically, studies using irradiation conduct behavioral experiments 2 month later (see Clark et al. 2008, Saxe et al. 2006, Snyder et al. 2005) and often fail to uncover an impairment in spatial learning. In two other studies cited above which showed the deficit, Deng et al. 2009 and Goodman et al. 2010, the training in MWM started 2-3 weeks after the beginning of the treatments. Evidently, the time between manipulating levels of neurogenesis and behavioral training can critically alter results and must be controlled for.

Lastly, immature DGCs and the DG itself are considered to play crucial role in pattern separation, and the involvement of the immature DGCs in hippocampal functions varies with the degree of ambiguity of the sensory input. In irradiated mice with reduced adult neurogenesis, deficits in contextual discrimination task are only seen when two highly similar contexts were used (Nakashiba et al. 2012). As such, seemingly minor differences in behavioral procedures (e.g., the complexity of the environment of a fear chamber or the size of environmental cues in the MWM) can have important impact on later behavioral output of the animal under investigation, and thus on the conclusions we derive regarding the function of adult generated DGCs in such experiments.

1.5.3 Adult neurogenesis and pattern separation

Since the seminal computational modelling work of David Marr (Marr 1971), the DG has been proposed to function as a pattern separator in the trisynaptic hippocampal circuitry allowing the formation of distinct memory representations of similar episodes. The sparseness of neural activity in the DG is proposed to underlie pattern separation (McNaughton 1991). The role of the DG in pattern separation has been experimentally proven in studies showing DG-specific lesion
or selective deletion of the NR1 subunit in the DG in mice, induced deficits in differentiating two similar contexts (Gilbert et al. 2001; McHugh et al. 2007).

In computational studies, adult neurogenesis was first modelled as a process continuously replacing mature DGCs and predicted to lead to faster clearance of old memories and more faithful recall of new memories (Deisseroth et al. 2004). Recently immature DGCs were found to be more excitable, plastic and preferentially incorporated into a spatial memory trace (Schmidt-Hieber et al. 2004; Ge et al. 2007; Kee et al. 2007). Computational work which took the enhanced plasticity of immature DGCs into account, proposed that immature DGCs reduce network interference from older memory traces (Wiskott et al. 2006).

Recent behavioral studies have confirmed the instructive role for adult neurogenesis in pattern separation. Niibori et al. (2012) found that pyramidal cell populations in CA3 encoding two similar contexts were less segregated in mice with adult neurogenesis reduced by TMZ. Similarly, two other studies respectively found deficits in discriminating two similar contexts in irradiated mice and in mice with tamoxifen-induced NR2B conditional knockout, a subunit of NMDA receptor underlying the enhanced plasticity in immature DGCs (Nakashiba et al. 2012; Kheirbek et al. 2012). Conversely the ability of contextual discrimination was improved in transgenic mice with enhanced survival of newborn DGCs (Sahay et al. 2011).

Notably, how much immature DGCs partake in hippocampal functions may depend on the need for pattern separation. In Sahay et al. 2011 and Nakashiba et al. 2012, the improvement or deficit of contextual discrimination was only seen when two highly similar contexts were used and there was no change (deficit or improvement) in the contextual fear memory formation.

1.5.4 Increased adult neurogenesis on behavior

The effect of increasing adult neurogenesis on hippocampus-dependent behaviors has always been an important aspect in adult neurogenesis research. This is based on the cognitive deficits observed in animal models with reduced adult neurogenesis, such as failure in efficacy of antidepressants, deficits in hippocampus-dependent learning or memory retention and deficits in discriminating similar contexts. An intuitive idea is that increasing adult neurogenesis would result in the opposite; such as mitigation of depression and promotion of hippocampal functions. Indeed, in early studies physiological manipulations increasing adult neurogenesis via
environment enrichment or voluntary running, were found to improve hippocampus-dependent contextual fear learning in CFC (Greenwood et al. 2009; Kohman et al. 2012; van Praag et al. 1999b) and spatial learning in MWM (Kempermann et al. 1997; van Praag et al. 1999a, 2005).

Especially, increased adult hippocampal neurogenesis result in improved pattern separation as predicted by previous theoretical works (Wiskott et al. 2006). For example, running was found to improve spatial pattern separation in mice (Creer et al. 2010). Environmental enrichment improved contextual discrimination in rats (Barbelivien et al. 2006; Woodcock and Richardson 2000). In a recent study using an inducible BAX knockout mouse model (iBax) to increase adult neurogenesis (Sahay et al. 2011) a significant improvement was observed in contextual discrimination task of two highly similar contexts one of which was associated with shocks during training.

In addition to facilitate the formation of new memories, recent studies have linked adult hippocampal neurogenesis with clearance of old memory. Previous studies in our group has shown that post-training voluntary running increases adult neurogenesis and degrades previously acquired spatial or contextual fear memory (Akers et al. 2014), and facilitates reversal learning of a new platform location in MWM (Unpublished data). Moreover the increased adult neurogenesis is causally correlated to the running-induced improvements as this effect is not observed in GCV-treated Nestin-tk runner mice, in which the running-induced increase of neurogenesis is normalized to saline-treated non-runners levels by the GCV treatment. (Unpublished data).

Notably, some contradicting results were given by these studies. First, learning in CFC and MWM is improved in some previous paper using voluntary running or environmental enrichment to increase neurogenesis (Greenwood et al. 2009; Kempermann et al. 1997; van Praag et al. 1999a, 1999b, 2005) but not in the runner mice studied by our group or iBax mice in Sahay et al. 2011. Second, improved reversal learning in MWM was shown in runner mice by our group and was absent in the iBax mice of Sahay et al. 2011.

Because of the complex and different mechanisms produced by these models, it is difficult to solve these discrepancies. The problem for running, environmental enrichment and other non-transgenic methods is that they are not specific. As mentioned before, voluntary running can promote neuronal plasticity through a BDNF-mediated mechanism (Gómez-Pinilla et al. 2002)
and increase cerebral protein synthesis (Nadel et al. 2013). Indeed, voluntary running was reported to enhanced hippocampus-independent cued Pavlovian fear conditioning (Falls et al. 2010) and amygdala activation (Burghardt et al. 2006).

For iBax mice, the problem is that apoptosis is eliminated in Bax-deficient newborn cells which is necessary to remove erroneous cells with abnormal development. In Bax knockout mice abnormal migration of new-born DGCs were found (Sun et al. 2004). Particularly in the Sahay et al. 2011 study, DGCs with Bax deletion showed more tertiary branching. Therefore in ibax mice, functional normality of newborn cells is not guaranteed.

To explore the functional significance of increasing neurogenesis, more studies using new pro-neurogenesis transgenic models must be done, which is the purpose of this study.

1.5.5 Summary: two mechanisms

During the process of integration and maturation, immature DGCs exert unique functions via anterograde and retrograde mechanisms. In anterograde, hippocampus-dependent learning is improved through the intrinsic properties of young neurons being more excitable and plastic. Computational modelling works have proposed a role for immature neurons in reducing interference from existing memory networks (Wiskott et al. 2006). Some evidence suggested that NR2B-mediated EPSC may contribute to the extra plasticity of immature DGCs (Ge et al. 2007) as knocking-out NR2B specifically in neural progenitors resulted in impaired novelty exploration and contextual discrimination in adult mice (Kheirbek et al. 2012). Moreover, 4-week old DGCs are less sensitive to GABAergic input owing to the late arrival of perisomatic GABAergic input which mediates feedforward inhibition (Ge et al. 2008; Espósito et al. 2005). As a result, studies showed that 4-week and 6-week old DGCs are preferentially incorporated into the hippocampal memory trace (Kee at al. 2007). Selective deletion of immature DGCs degraded memory acquired before the deletion but not memory acquired afterwards (Arruda-Carvalho et al. 2011).

Knocking-out of neurogenesis also rendered population coding of two different contexts in CA3 less segregated (Niibori et al. 2012) and impaired pattern separation (Clelland et al. 2009).

In retrograde, old memory is degraded by young DGCs through their integration remodeling the local network. It has been shown that, most of the filopodia on the immature DGCs target preexisting axon boutons that are already connected to other neurons (Toni et al. 2007).
Similarly, the boutons on mossy fibers of new DGCs form synapses with the dendritic shaft near or directly on thorny excrescences of mature DGCs (Toni et al. 2008, Faulkner et al. 2008). Because immature DGCs are more excitable and more plastic, it is plausible that they may be more likely to replace the existing synapses via mechanisms of spiking time dependent plasticity, and thus alter the information stored in these old connections. As shown in previous study from our group, post-training voluntary running which increases proliferation and survival of new-born DGCs induces forgetting of recent contextual fear and spatial memories (Akers et al. 2014). This process is important to degrade old memories and maintain the capacity to encode new memories as predicted by previous computational work (Deisseroth et al. 2004) and confirmed by studies in our lab showing increased adult neurogenesis improves reversal learning in runner mice (Unpublished data).

1.6 p53 and neurogenesis

p53 is one of the most important tumor repressing transcription factors. To repress tumor and exert other functions such as regulating neurogenesis, p53 functions as a master switch controlling cell survival and death, growth and arrest in response to cellular stress. p53 is expressed at high level in developing brains and the neurogenic niches of adult brains (Meletis et al. 2005; van Lookeren Campagne and Gill 1998). Upon activation, p53 initiates a wide range of protective processes, such as cell cycle arrest, apoptosis and senescence through its transcriptional regulation of numerous p53 target genes.

1.6.1 p53 and cell cycle control

In response to cellular stress, activation of p53 may lead to cell cycle arrest at both G1/S and G2/M transits through induction of cell cycle-dependent kinase (CDK) inhibitors and repression of cell cycle phosphatase (Fig. 5), allowing time for repairing the genome. The first set of p53-regulated cell cycle inhibitors, namely p21Cip1 and p27Kip1, are involved in both G1 and G2 checkpoint control (van Lookeren Campagne and Gill 1998). They can inhibit both CDK2/cyclin E and Cdc2/cyclin B complexes and induce cell cycle arrest at G1 and G2 (Deng et al. 1995; Flatt et al. 2000; Medema et al. 1998; Coats et al. 1996; Payne et al. 2008). The second set of p53-regulated cell cycle inhibitors, namely 14-3-3σ and Gadd45, target Cdc2/cyclin B complex
and regulate G2 checkpoint in specific. Gadd45 binds to Cdc2 inhibiting the formation of Cdc2/cyclin B complex (Jin et al. 2000) and 14-3-3α inhibit phospho-Cdc25C and sequester Cdc2 from translocating into the nucleus (Chan et al. 1999). Two cell cycle phosphatases Cdc25A and Cdc25C responsible for activating CDK2/cyclin E and Cdc2/cyclin B respectively are repressed by p53 through direct binding to their promoter regions (Rother et al. 2007; St Clair et al. 2004).

Some of these mechanisms have been verified in the context of adult neurogenesis. In p53-knockout (p53KO) mice, cell proliferation in the SVZ and of neurosphere-forming cells in vitro was increased, concomitant with reduced expression of p21Cip1 and p27Kip1, although the expression of Gadd45 was reported normal (Aremesilla-Diaz et al. 2009; Meletis et al. 2006; Gil-Perotin et al. 2006). In the forebrain of p21-deficient mice higher proportion of dividing NSCs out of total NSCs were found (Kippin et al. 2005). Besides, p27Kip1 is likely to be involved in transit from transient amplifying precursors (TAPs) to neuroblasts in SVZ, as there were more TAPs but less neuroblasts in p27Kip1-null mice compared with controls (Doetsch et al. 2002). These results together indicate that in the absence of p53 and reduced expression of p21Cip1 and p27Kip1, more NSCs proliferate and neural progenitors commit more cell cycles before postmitotic stages.
Figure 5. Activation of p53 leads to cell cycle arrest at G1/S and G2/M transits through the induction of p21Cip1, p27Kip1, Gadd45 and 14-3-3α and repression of Cdc25C and Cdc25A.

1.6.2 p53 and apoptosis

When the impairment or instability caused by cellular stress is irreversible, p53 induces apoptosis. In one way, p53 is able to activate both intrinsic and extrinsic cell death pathways by inducing transcription of pro-apoptotic target genes, such as Bax, Apaf-1, Puma, Pten and Caspase-1 (reviewed in Pietsch et al. 2008). In the other way, p53 can induce transcription-independent apoptosis through direct interaction with mitochondrial BCL-2 family protein, such as pro-apoptotic BAK, Bax and Bid. Evidences suggested that upon translocation to the mitochondria, p53 can release BAK and Bax from their respective inhibitory partner MCL-1 and BCL-x1 allowing them to oligomerize and induce cytochrome C release from the mitochondria (Leu et al. 2004; Chipuk et al. 2004).

The function of p53 in regulating cell death has been studied in the context of adult neurogenesis. In the neurospheres derived from the SVZ of p53KO mice, irradiation-induced apoptosis was almost abolished, compared to over 40% dying cells seen in WT control (Armesilla-Díaz et al. 2009). In 53KO mice the cells in the SGZ committing PCD were reduced by 50% compared to WT mice (Cancino et al. 2013).

The function of p53 is antagonized by the truncated forms of p63 and p73 (ΔNp63 and ΔNp73) and facilitated by p63 and p73 with trans-active domains (TAp63 and TAp73). The remaining cell death in vivo in Cancino et al. 2013 may be attributed to the compensation from TAp63 and TAp73 and other p53-independent pathways. In the neurospheres derived from the SGZ of WT mice, only ΔNp63 but not TAp63 was detected (Cancino et al. 2013). However whether TAp63 or TAp73 would be elevated after the deletion of p53 in the SGZ neural progenitors is unknown.

1.6.3 p53 and postmitotic development

p53 can regulate the development of postmitotic cells through the activation of mTORC-1 (the mammalian target of rapamycin complex 1) pathway, which is known to induce mRNA translation and cell growth (Fingar et al. 2002, 2004). Kim and colleagues found that mTORC-1
activated by knocking down DISC1 in adult neural progenitors using retrovirus-delivered shRNA led to increased cell growth (indicated by increased cell body size, dendritic length and complexity) which were rescued by pharmacological inhibition of mTORC-1 with rapamycin (Kim et al. 2009, 2012). p53 can repress mTORC-1 pathway by up-regulating the expression of Sestrin 1, Sestrin 2, AMPK β1 (AMP-activated protein kinase β1) and Pten (Feng et al. 2007; Budanov and Karin 2008). Indeed the cDNA microarray data of the NSCs derived from p53KO mice revealed a significant down-regulation of Sestrin 2 (Meletis et al. 2006). Thus deletion of p53 may result in mTORC-1 activation.

1.7 Hypothesis and aims

Previous studies using tamoxifen (TAM)-inducible CreER\textsuperscript{T2}-based transgenic system have shown that the system is advanced in providing fine temporal and spatial control, an attribute preferable in behavioral studies (Arruda-Carvalho et al. 2011;Imayoshi et al. 2008; Lagace et al. 2007, DeCarolis et al. 2013). Enlightened by these studies, we constructed an inducible p53 conditional deletion model by crossing p53 flox mice with Nestin-CreER\textsuperscript{T2} mice on a C57BL/6 background. As such, p53 deletion would only occur in the Nestin+ neural progenitors of double transgenic mice when TAM is given. Based on previous findings, we hypothesize that adult hippocampal neurogenesis would increase after inducible p53 conditional deletion by means of promoting cell proliferation and survival.

Our next mission is to use this model to study both the anterograde and retrograde effects of increased adult neurogenesis on hippocampus-dependent behaviors. The anterograde effect is defined as the altered ability to acquire and store new memories after the manipulation of neurogenesis. We would first inject TAM to induce p53 conditional deletion in the transgenic mice and then subject them to the training and testing of CFC 4 weeks or 8 weeks later. Considering contradicting results were given by previous studies, we predict a moderate to minor increase of freezing in the mice with p53 conditional deletion.

The retrograde effect is defined as how altered neurogenesis interacts with the memories encoded before the manipulation of neurogenesis, we would first train the transgenic mice in CFC and then inject them with TAM. 4 weeks later they would be tested in the conditioning
chamber. Previous studies in our group has established solid link between running-induced increased neurogenesis and the degradation of previously acquired memory (Akers et al. 2014 and unpublished data). Therefore we anticipate similar effect in our mice with p53 conditional deletion.
Materials and Methods

2.1 Animals

All mice lines were maintained on a C57BL/6 background (Taconic Farms), bred and housed in group in the animal facilities at the Hospital for Sick Children with continuous access to clean food and water.

Nestin-CreERT2 mice

The Nestin-CreERT2 mice we used in this study corresponds to line 4 in Imayoshi et al. 2008. They express TAM-inducible Cre recombinase (Cre-ERT2) under the control of a nestin promoter as described previously (Imayoshi et al. 2008).

P53 flox mice

In p53 flox mice (The Jackson Labrotary), exons 2-10 of Trp53 gene are flanked by loxP sites in this conditional targeted mutation.

iKO-p53-/-, iKO-p53+/- and CTR mice

Nestin-CreERT2 mice were crossed with homozygous p53 flox mice to generate Nestin-CreERT2 x p53 flox/wildtype (wt) mice (inducible p53 heterozygous deletion, or iKO-p53+/-). iKO-p53+/- mice were crossed with CTR mice to produce Nestin-CreERT2 x p53 flox/flox mice (inducible p53 knockout or iKO-p53-/-). CreERT2-negative littermates (p53 flox or p53 flox/wt) were used as control (CTR).

2.2 TAM administration

Every 30 mg of TAM (stigma) was dissolved in 100ml ethanol and suspended in 900ml sunflower oil. TAM (180 mg/kg) was administrated intraperitoneally once per day, for 5 consecutive days started when mice were 8-week old.

2.3 Immunohistochemistry

Mice were perfused transcardially with phosphate-buffered saline (PBS, 0.009 M) and paraformaldehyde (PFA, 4% w/v) 1 d after the behavioral experiments. Brains were dissected,
post-fixed overnight in 4% PFA at 4°C, and stored in 0.009 M PBS at 4°C before processing. The brains were sliced into coronal sections (50 µm) using a vibratome (VT1200S; Leica). All sections were deparafinized, heated for antigen retrieval (at 100°C for 40 min) and blocked with hydrogen peroxidase (1% w/v) and normal donkey serum (4% w/v). Sections were then incubated for 2 overnights in rabbit polyclonal anti-Doublecortin (DCX) antibody (1: 1000, cell signaling) and then 2 h in biotinylated secondary antibody (1:1000; Jackson Immunoresearch Laboratories) followed by signal amplification with Vectastain Elite ABC kit (Vector Laboratories), and biotin-conjugated tyramide signal amplification (TSA, 1:100). Sections were then re-blocked with 3% hydrogen peroxidase and 4% Ki-67 normal donkey serum with rabbit polyclonal anti-rabbit IgG antibody (1:100, sigma) before being incubated overnight with rabbit polyclonal anti-Ki67 antibody (1:200, Abcam) and 2 h with HRP-conjugated secondary antibody (1:300; Jackson Immunoresearch Laboratories). DCX, Ki-67 and nucleus signals were visualized with Alexa Fluor® 568 conjugated streptavidin (1:300), FCM-conjugated TSA (1:100) and Hoechst (1:1000), respectively.

Ki-67+ cells and DCX+ cells were quantified with fluorescent microscope (Olympus BX61) under 60x objective manually and normalized to the area of GCL of each section. Statistical comparison of cell densities was made between iKO-p53−/− or iKO-p53+/* mice and CTR mice using Statistica 10 (Statsoft).

2.4 Behavior test

Contextual fear conditioning was performed in a stainless-steel conditioning chamber (31 x 24 x 21 cm; MED Associates). The floor was made of stainless-steel shock grid bars (diameter 3.2 mm) spaced 7.9mm apart. A stainless-steel drop pan odorized with 70% ethyl alcohol was positioned beneath the grid bars to provide a background odor. The front, back and top of the conditioning chamber were made of transparent acrylic, and the left and right sides were made of modular aluminum.

For the study of post-training deletion, training of contextual fear conditioning began when the mice were 8 weeks old. iKO-p53−/− or iKO-p53+/* with CTR mice were placed in a chamber for 3 minutes and a shock (0.7 mA) lasting for 2 seconds was delivered at 150 s. After the training, mice received 5 daily injections of TAM (180mg/kg). 4 weeks after mice received the last
injection of TAM, they were returned to the chamber where they had received shock and stayed for 5 minutes, the percentage of time of freezing was recorded.

For the study of pre-training deletion, training of contextual fear conditioning began 8 weeks after mice received the last injection of TAM. iKO-p53+- and CTR mice were placed in a chamber for 3 minutes and a shock (0.5 mA) lasting for 2 seconds was delivered at 150 s. 24 hours after the training, mice were returned to the chamber where they had received shock and stayed for 5 minutes, the percentage of time of freezing was recorded.
Results

Previous studies employed a variety of methodologies manipulating adult neurogenesis to investigate its functions in hippocampus-dependent learning and memories. However, a majority of these methods decrease neurogenesis. With the available pro-neurogenesis models, which are voluntary running, environmental enrichment and iBax, conducive effect of increasing adult neurogenesis on hippocampal functions has been demonstrated yet much unknown remains (Kempermann et al. 1997; Sahay et al. 2011; van Praag et al. 2005). To potentiate future studies on increasing adult neurogenesis, in present study we create a new pro-neurogenesis transgenic model with inducible p53 conditional deletion by crossing Nestin-CreERT2 mice with p53 flox mice.

The Nestin-CreERT2-mediated recombination has been demonstrated to be highly specific (Arruda-Carvalho et al. 2011; Imayoshi et al. 2008). At 4 weeks of age, CreERT2 expression was restricted to Nestin+ progenitors in the SVZ and SGZ of Nestin-CreERT2 mice. Recombinant cells were detected in the SVZ and SGZ of Nestin-CreERT2/ROSA26-LacZ mice but not in Nestin-CreERT2/ROSA26-LacZ controls. The recombination efficiency in the SGZ was shown in different conditions. In Imayoshi et al. 2008, with 12-day BrdU administrated at 10 dpi of TAM, 66% BrdU+ cells are recombinant cells expressing reporter gene. In Arruda-Carvalho et al. 2011, at 7 wpi of TAM, 86% dividing Ki-67+ cells are recombinant cells. Because TAM doesn’t affect Type-III neuroblasts which may remain in cell cycle at earlier time point after TAM, the actual recombination rate might approximate the number given in Arruda-Carvalho et al. 2011. Therefore in our system, TAM-induced p53 deletion should be specific in Nestin+ progenitors with high recombination efficiency.

To characterize this transgenic model, we examined potential change in hippocampal neurogenesis and hippocampus-dependent behaviors at 4 wpi and 8 wpi in iKO-p53−/− and iKO-p53+/− mice. Previous studies in IEG expression and electrophysiological properties of adult-born DGCs suggested that the time window of functional integration of them is 3-7 weeks (Ge et al. 2007; Kee et al. 2007). Behavioral effects manifested 4-8 weeks after the manipulation of neurogenesis across studies using different experimental paradigms (Garthe et al. 2009; Saxe et al. 2006; Wang et al. 2008; Sahay et al. 2011). Therefore we choose 4 wpi and 8 wpi in our study.
To visualize neurogenesis, early neuronal marker DCX was stained to indicate the number of immature neurons, and Ki-67 was stained to visualize dividing cells. We found increased immature neurons and dividing cells in both iKO-p53<sup>−/−</sup> and iKO-p53<sup>+/−</sup> mice at 4 wpi.

To reveal the anterograde and retrograde effect of p53 deletion on behaviors, we inject TAM in iKO-p53<sup>+/−</sup> and iKO-p53<sup>−/−</sup> mice with littermate control either before or after being trained in CFC. First, iKO-p53<sup>−/−</sup> mice receiving 5-day TAM one day after the training showed decreased freezing behavior when they were tested at 4 wpi.

Second, iKO-p53<sup>+/−</sup> mice receiving TAM 8 weeks before training showed trend of increased freezing behavior when they were tested one day after training, resembling the learning-facilitating effect found in runner mice (Greenwood et al. 2009; Kohman et al. 2012; van Praag et al. 1999b).

In summary, we found increased proliferation and survival of newborn neurons in adult iKO-p53<sup>+/−</sup> and iKO-p53<sup>−/−</sup> mice, and in iKO-p53<sup>+/−</sup> mice, this change in neurogenesis was concomitant with enhancing ability to form new and clear old contextual fear memory.

### 3.1 Experiment 1: Ault neurogenesis was increased following p53 deletion

iKO-p53<sup>−/−</sup>, iKO-p53<sup>+/−</sup> and Cre<sup>ERT2</sup> negative control (CTR) mice received 5 daily TAM injection and were perfused 4 weeks after the last injection. To characterize neurogenesis, we used DCX as an early neuronal marker, and Ki-67 as a marker for cell proliferation. DCX is expressed in migratory cells and found to be important in hippocampal lamination (Corbo et al. 2002). In SGZ neurogenesis, DCX expression starts from Type IIb progenitors, persists through early postmitotic stage and ceases before full maturation (Jinno et al. 2011; Kronenberg et al. 2003; Spampanato et al. 2012). Slice recording showed that DCX<sup>+</sup> cells in the SGZ generated small Na<sup>+</sup> currents and few action potentials in response to injected current confirming the immature phenotype (Klempin et al. 2011; Spampanato et al. 2012). Ki-67, on the other hand, is express in all phases of cell cycles excluding G<sub>0</sub> (Endl and Gerdes, 2000; Scholzen and Gerdes, 2000). It has been shown to be a reliable endogenous marker for cell proliferation in the context of adult neurogenesis (Kee at al. 2002).
In iKO-p53+/− mice, we found significantly more DCX+ cells (1238.69 vs. 1157.48 /mm², n= 4, unpaired t test, t(6)= 2.98, p<0.05) and Ki-67+ cells (1175.10 vs. 763.46 /mm², n= 4 iKO-p53+/−, 5 CTR, unpaired t test, t(7)= 2.96, p<0.05) (Fig. 6). In iKO-p53−/− mice, we also found significantly more DCX+ cells (1337.31 vs. 997.04 /mm², n= 4, unpaired t test, t(6)= 5.76, p<0.01) and Ki-67+ cells (744.26 vs. 596.49 /mm², n= 5 iKO-p53−/−, 4 CTR, unpaired t test, t(7)= 2.96, p<0.05) (Fig. 7).
B

![Graph showing DCX positive cells/mm² for different genotypes](image)

**KO-p53+/− CTR** genotype

- DCX positive cells/mm²:
  - 0
  - 200
  - 400
  - 600
  - 800
  - 1000
  - 1200
  - 1400

**KO-p53−/−** genotype

- DCX positive cells/mm²:
  - * (significantly different)

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**Images showing Ki-67 and DAPI staining**

- **iKO-p53−/−**
- **CTR**

- Immunostaining for Ki-67 and DAPI
  - Scale bars: 50 µm and 200 µm
Figure 6. Adult neurogenesis was increased 4 weeks after TAM-induced p53 deletion in iKO-p53+/− mice. (A) DCX+ cells in the DG of iKO-p53+/− (upper panel) and CTR (lower panel) mice. Scale bar, 200 μm. Insert, high magnification. Scale bar, 50 μm. (B) DCX+ cells were increased (*p<0.05). (C) Ki-67+ cells in the DG of iKO-p53+/− (upper panel) and CTR (lower panel) mice. Scale bar, 200 μm. Insert, high magnification. Scale bar, 50 μm. (D) Ki-67+ cells were increased (*p<0.05).
iKO-p53\(-/-\)

DCX

DAPI

CTR

DCX

DAPI
B

![Bar graph showing the number of DCX positive cells/mm² for CTR and iKO-p53−/− genotypes. The graph indicates a significant difference (**) between the two groups.](image)

C

![Image showing Ki-67 and DAPI staining in iKO-p53−/− and CTR genotypes.](image)
Figure 7. Adult neurogenesis was increased at 4 wpi in iKO-p53⁻/⁻ mice. (A) DCX⁺ cells in the DG of iKO-p53⁻/⁻ (upper panel) and CTR (lower panel) mice. Scale bar, 200 μm. (B) DCX⁺ cells were increased (**p<0.01). (C) Ki-67⁺ cells in the DG of iKO-p53⁻/⁻ (upper panel) and CTR (lower panel) mice. Scale bar, 200 μm. (D) Ki-67⁺ cells were increased (*p<0.05).

Based on the assumption that the neurogenesis status of CTR lines and the experimental conditions in two experiments are consistent, Ki-67⁺ and DCX⁺ cell density was normalized to CTR mice to compare neurogenesis between iKO-p53⁻/⁻ and iKO-p53⁺/⁻ mice (Fig. 8). There is no difference in the normalized Ki-67⁺ cell density (1.25 vs. 1.54, unpaired t test, t(7)= 1.73, p>0.05) and significantly more DCX⁺ cell density in iKO-p53⁻/⁻ mice (1.34 vs. 1.07, unpaired t test, t(6)= 12.28, p<0.001)
Figure 8. Ki-67+ and DCX+ cell density was normalized to control and compared between iKO-p53+/− and iKO-p53−/− mice. There is (A) no difference in the Ki67+ density (p>0.05) and (B) significant more DCX+ cells in the ip53 mice (**p<0.001).
3.2 Experiment 2: Increased adult neurogenesis degraded previously acquired contextual fear memory.

This experiment was to evaluate the impact of increased adult neurogenesis on previously encoded memory. Computational studies have proposed a role for hippocampal neurogenesis to degrade old memories and maintain capacity to encode new memories (Deisseroth et al. 2004). Previous paper in our group found a retrograde loss of spatial and contextual fear memories in mice housed with a running wheel for 4 weeks (Akers et al. 2014). To explore whether increased adult neurogenesis induced by p53 deletion would produce similar effect, iKO-p53+/−, iKO-p53−/− and CTR mice were trained in the contextual fear learning paradigm (0.7 mA, one shock) 1 day before TAM injection. 4 weeks after the last injection, mice were placed back to the conditioning context and the memory retention was accessed. We found a significantly less freezing in iKO-p53+/− mice (n= 9 iKO-p53+/+, 14 CTR, unpaired t test, t(21)= 2.68, p<0.05) and a trend of less freezing in iKO-p53−/− mice (n= 11 iKO-p53−/+, 14 CTR, unpaired t test, t(23)= 1.44, p>0.05) compared with CTR (Fig. 9).
Figure 9. Mice with post-training p53 deletion showed reduced contextual fear memory when being tested 4 week after TAM. (A) The reduction was significant in iKO-p53+/− mice (*p<0.05) but (B) insignificant in iKO-p53−/− mice (p>0.05) compared with control.

3.3 Experiment 3. Increased adult neurogenesis may facilitate contextual fear learning

Although young DGCs are found to be more plastic and preferentially incorporated into hippocampal memory network (Ge et al. 2007; Kee et al. 2007), with respect to whether elevated adult neurogenesis facilitates new hippocampus-dependent learning, previous studies have presented conflicting findings (Kempermann et al. 1997; van Praag et al. 2005; Sahay et al. 2011). To explore whether increased adult neurogenesis induced by p53 deletion provides anterograde facilitation of new learning, heterozygous p53 conditional deletion was induced by TAM injection in iKO-p53+/− but not in CTR mice. 8 weeks later, they were subjected to a contextual fear learning paradigm. One day after the training, they were placed back to the conditioning chamber and their fear memory was accessed by scoring the time animals spent freezing. With limited number of subjects (8 iKO-p53+/−, 10 CTR), we found a trend of increased
freezing behavior in iKO-p53$^{+/−}$ mice compared with CTR mice, though insignificant (unpaired t test, t(16)= 1.90, p=0.08) (Fig. 10).

Figure 10. Formation of contextual fear memory was enhanced insignificantly in iKO-p53$^{+/−}$ mice 8 weeks after TAM-induced p53 deletion (p>0.05).
Discussion

Adult hippocampal neurogenesis has been found to play an important role in many hippocampus-dependent behavior tasks, such as CFC, MWM, object-location and trace conditioning by functional knockout studies (Elbeltagy et al. 2009; Goodman et al. 2010; Deng et al. 2009; Shors et al. 2001; Snyder et al. 2009). Studies increasing neurogenesis are somewhat preliminary and yield conflicting data (van Praag et al. 2005; Kempermann et al. 1997; Sahay et al. 2011). One problem is that most of these studies were conducted using traditional non-transgenic manipulations which lack specificity. In this study we developed a new transgenic model that enables specific inducible increase of neurogenesis through TAM-dependent p53 deletion in Nestin-expressing cells. By IHC, we found an increase in number of newborn DCX positive neurons and dividing Ki-67 positive cells in the SGZ of mice with conditional p53 deletion (iKO-p53−/− and iKO-p53+/−) compared to CreERT2-negative littermate controls (CTR), indicating an increase in both cell proliferation and cell survival. To explore the behavioral effect of neurogenesis of conditionally deleting p53, iKO-p53−/−, iKO-p53+/− and CTR mice were subjected to two protocols of CFC experiments. First, to investigate the retrograde effect on memories encoded before manipulating neurogenesis, we trained adult mice in CFC and started TAM-induced deletion of p53 the next day. 4 weeks later, the mice were placed back into the shocking chamber to evaluate the fear memory. A significant decrease of freezing behavior was observed in iKO-p53+/− mice. Second, to investigate the anterograde effect on the formation of new memories, adult mice received TAM 8 weeks prior to CFC training and were tested 24h after. We found a trend of increased freezing behavior in iKO-p53+/− mice compared to controls.

4.1 Validity of Nestin-CreERT2 based transgenic system

Although our transgenic mice do not have a reporter gene to mark cells affected by TAM-induced p53 deletion, this Nestin-CreERT2 line corresponds to the transgenic line 4 in Imayoshi et al. 2008 and the specificity and recombination efficiency has been well established.

For specificity, firstly, CreERT2 was expressed only in neurogenic regions (SVZ and SGZ) in Nestin-CreERT2/ROSA-CFP (cyan fluorescent protein) mice (Imayoshi et al. 2008) and in Nestin-CreERT2/ROSA-LacZ mice (Arruda-Carvalho et al. 2011). Secondly, the CreERT2-mediated expression of the reporter gene was observed only in the progenitor cells in the SVZ and SGZ (Imayoshi et al. 2008; Arruda-Carvalho et al. 2011; Sahay et al. 2011). Thirdly, the
CreER\textsuperscript{T2}-mediated recombination was not observed in the brains of TAM-treated CreER\textsuperscript{T2}-negative littermates or oil-treated Nestin-CreER\textsuperscript{T2}/ROSA26-reporter mice in these studies (Imayoshi et al. 2008; Arruda-Carvalho et al. 2011).

For the recombination efficiency, Imayoshi et al. (2008) injected BrdU for 12 days starting at 10 dpi of TAM and the proportion of CFP\textsuperscript{+} cells in BrdU\textsuperscript{+} cells in the SGZ was 66%. The recombination efficiency was also examined by Arruda-Carvalho et al. (2011) in Nestin-CreER\textsuperscript{T2}/ROSA-LacZ mice, which showed that at 7 wpi of TAM 86% of dividing Ki-67\textsuperscript{+} cells exhibited CreER\textsuperscript{T2}-mediated LacZ expression. The latter is more relevant to the present study for two reason. First, they used Ki-67 as the marker for dividing cells, same as in the present study. Second, 7 wpi is more proximate to the time points in the present study (4 wpi and 8 wpi). In Imayoshi et al. 2008, at 10 dpi of TAM when BrdU injection started, some nestin\textsuperscript{+} progenitors were still quiescent and nestin\textsuperscript{-} neuroblasts (unaffected by TAM) may persist. Thus, in the present study we estimated that over 80% DCX\textsuperscript{+} and Ki-67\textsuperscript{+} cells are recombinant cells at 4 wpi and 8 wpi.

### 4.2 p53 deletion increases adult neurogenesis

In both iKO-p53\textsuperscript{-/-} and iKO-p53\textsuperscript{+/-} mice sacrificed 4 weeks after TAM treatment, there was a significant increase in DCX\textsuperscript{+} and Ki-67\textsuperscript{+} cells. Interestingly, iKO-p53\textsuperscript{+/-} mice had 53% increase of Ki-67\textsuperscript{+} cells and more modest 7% increase of DCX\textsuperscript{+} cells whereas iKO-p53\textsuperscript{-/-} had 25% increase of Ki-67\textsuperscript{+} cells and 34% increase of DCX\textsuperscript{+} cells. To compare neurogenesis between iKO-p53\textsuperscript{-/-} and iKO-p53\textsuperscript{+/-} mice, Ki-67\textsuperscript{+} and DCX\textsuperscript{+} cell density was normalized to littermate controls (based on the assumption that the neurogenesis status in the littermate control in the two experiments is not different). There is no difference in the normalized Ki-67\textsuperscript{+} cell density and significantly higher DCX\textsuperscript{+} cell density in iKO-p53\textsuperscript{-/-} mice.

Given that over 50% of maximal BrdU\textsuperscript{+} cells die during the first 2 dpi (of BrdU) in mice (Mandyam et al. 2007; Sierra et al. 2010), the increase of Ki-67\textsuperscript{+} cells indicates a combined effect of increased cell proliferation and survival of neural progenitors. The similar increase in Ki-67\textsuperscript{+} cells in iKO-p53\textsuperscript{-/-} and iKO-p53\textsuperscript{+/-} mice may thus indicate that the function of p53 in neural progenitors is sensitive to p53 deletion so that the deletion in one allele produces comparable effect to the deletion in both alleles.
DCX is commonly used as an early neuronal marker. During this stage, newborn cells decrease by 25% of maximal number from 2 dpi to 2 wpi (Mandyam et al. 2007; Sierra et al. 2010). Because apoptotic cells doesn’t express DCX (Sierra et al. 2010), DCX+ number reflects survival of newborn DGCs. Therefore the difference in DCX+ number in the iKO-p53−/− and iKO-p53+/− mice indicates that heterozygous p53 deletion is less efficient than homozygous p53 deletion in promoting the survival of immature neurons.

4.3 Comparison to other pro-neurogenesis methods

Earlier experiments demonstrated that adult neurogenesis is increased by physiological stimuli, for example voluntary running, environmental enrichment and hippocampus-dependent learning. Since then voluntary running and environmental enrichment are commonly used to increase neurogenesis in behavior studies. Typically, 4-week housing with access to a running wheel yields over 100% increase of BrdU+ cells sacrificed at 4 wpi of BrdU and nearly 100% increase of BrdU+ cells sacrificed at 1 dpi in mice (van Praag et al. 1999a, 1999b, 2005). In comparison, 4-week environmental enrichment does not produce increase of BrdU+ cells in mice sacrificed at 1 dpi but 80-90% increase of BrdU+ cells in mice and rats sacrificed at 4 wpi of BrdU (Kempermann et al. 1997; van Praag et al. 1999a; Nilsson et al. 1999). These results indicate that 4-week running increases proliferation and long-term survival of newborn DG cells by one fold whereas 4-week environmental enrichment also increases survival of newborn DG cells by one fold but does not affect cell proliferation.

The only well-characterized transgenic model with inducible increase of adult neurogenesis is iBax (Nestin-Cre/ Bax flox) mice (Sahay et al. 2011). Bax is a pro-apoptotic mitochondrial BCL-2 family protein (Mitchell et al. 2000; Zhang et al. 2000). It has been found to be necessary in the apoptosis during adult neurogenesis as TUNEL+ (Terminal deoxynucleotidyl transferase dUTP nick end labeling) cells are not observed in the DG of adult Bax knockout mice (Sun et al. 2004). In line with these results, conditional Bax ablation increased DCX+ cells by 70-80% without affecting cell proliferation at 8 wpi of TAM treatment (Sahay et al. 2011).

Compared to these models, iKO-p53−/− mice showed moderate increase of immature neurons and proliferating cells whereas iKO-p53+/− mice showed moderate increase of proliferating cells but only a small increase of immature neurons. The magnitude of alteration is reminiscent of previous studies examining adult neurogenesis in p53KO mice. Cancino et al. (2013) uncovered
a 20% increase of DCX$^+$ cells and dividing Sox2$^+$ cells (labelling NSC and astrocyte) in the DG. Interestingly, this study also showed an over 50% reduction in apoptotic cells, which is distinct from the lower increase in DCX$^+$ cells and BrdU$^+$/NeuN$^+$ cells.

Nevertheless the amount of increase in adult neurogenesis is not the only criterion for a good model in neurogenesis study. Our model is much more specific to neurogenesis compared to traditional non-transgenic methods, and compared to iBax model, it has less effect on apoptosis potentially permitting removal of abnormal neurons.

First, traditional non-transgenic methods, such as voluntary running and environmental enrichment, produce many effects unspecific to adult neurogenesis. Running could induce pro-growth effect through multiple mechanisms, for example through promoting angiogenesis (van Praag et al. 2005), BDNF secretion (Neeper et al. 1995) and protein synthesis (Nadel et al. 2013) in the hippocampus as well as in other brain regions. Similarly, environmental enrichment enhances increases BDNF secretion in multiple brain regions (Ickes et al. 2000). Meshi et al. (2006) found that spatial learning enhanced by environmental enrichment did not depend on adult neurogenesis, which was evident in irradiated mice with completely abolished neurogenesis.

Second for the iBax model, the 70-80% increase of DCX$^+$ cells resulted from complete block of apoptosis. Comparing the peak number of BrdU-labelled cells at 4 wpi in iBax mice to wildtype mice, the lost cells were exactly 70-80% of the maximal number (Mandyam et al. 2007; Sierra et al. 2010). Without competition, functionally abnormal neurons are able to survive, as Bax knockout mice exhibited abnormal migration of newborn DGCs (Sun et al. 2004).

p53 is expressed at high levels in differentiating progenitors and only at low levels in unstressed postmitotic neurons (Rogel et al. 1985; Louis et al. 1988; Tendler et al. 1999), implying a specific effect of inducible conditional p53 deletion on adult neurogenesis. Meanwhile, the increase of DCX$^+$ cells is less in iKO-p53$^{-/-}$ mice and a lot less in iKO-p53$^{+/-}$ mice compared with iBax mice, indicating a moderately enhanced survival in our model. Therefore with smaller magnitude, our model have advantages in specificity and quality of newborn DGCs.
4.4 p53 deletion facilitates contextual fear learning

Contextual fear conditioning is a classic hippocampus-dependent learning task in which the formation and retention of a context-specific fear memory is tested (McEchron et al. 1998; Phillips et al. 1994). An anterograde deficit in contextual fear learning has been demonstrated in rodent models with knockdown of adult hippocampal neurogenesis by irradiation (Drew et al. 2010; Snyder et al. 2009; Saxe et al. 2006; Wojtowicz et al. 2008), 5-Fluorouracil (Elbeltagy et al. 2009) and transgenic methods, such as Nestin-CreER\textsuperscript{T2}/ROSA26-DTA (Imayoshi et al. 2008), GFAP-tk (Saxe et al. 2006) and galectin-1 knockout mice (Sakaguchi et al. 2011). Conversely, contextual fear learning is enhanced in running mice (Greenwood et al. 2009; Kohman et al. 2012; van Praag et al. 1999b). Similarly, spatial learning in MWM is compromised with reduced adult neurogenesis (Deng 2009; Garthe et al. 2009; Goodman et al. 2010) and enhanced with increased adult neurogenesis (Kempermann et al. 1997; van Praag et al. 2005). In contradiction to some of these results, Sahay et al. 2011 found no improvement in CFC and MWM in iBax mice.

In our hands, iKO-p53\textsuperscript{+/−} mice exhibit a trend of enhanced contextual fear learning compared with controls, in line with previous experiments using running or environmental enrichment to increase neurogenesis. At this stage, the difference is not yet statistically significant possibly due to the limited sample size.

The question arises that since p53 deletion promotes contextual fear learning, why is this not observed when Bax is deleted given the larger number of new neurons in iBax mice? As mentioned above, Bax deletion is unique as it promotes cell survival by completely blocking apoptosis. In contrast, running and environmental enrichment increase cell survival via neurotropic mechanisms, and p53 deletion blocks apoptosis to a lesser degree. Therefore a plausible reason for this discrepancy is that in iBax mice, some damaged or abnormal neurons were allowed to survive and not able to promote the acquisition of contextual fear memory.

In our experiment, training in CFC was conducted at 8 wpi allowing increase of newborn neurons of 0-8 weeks old. Among these neurons, 4-7 weeks old newborn neurons are hypothesized to produce an anterograde effect through their hyper- excitability and plasticity (Schmidt-Hieber et al. 2004; Ge et al. 2007). High-frequency stimulation of the medial perforant path elicits a newborn DGC-dependent LTP in the DG which is abolished in irradiated mice,
TMZ-treated mice and GCV-treated GFAP-tk mice (Garthe et al. 2009; Saxe et al. 2006; Snyder et al. 2001; Wang et al. 2008), but enhanced in chronic fluoxetine-treated mice (Wang et al. 2008). These treatments were either chronic (>4 weeks) or given at least 4 weeks before behavior studies. Indeed, 4-week and 6-week old newborn DGCs are found to be preferentially incorporated into hippocampal memory trace formed in MWM (Kee et al. 2007) and post-training deletion of these neurons degrade hippocampus-dependent contextual fear and spatial memories (Arruda-Carvalho et al. 2011). These studies indicated that 4-7 week old DGCs enhance synaptic plasticity in the DG and directly partake in the formation of contextual fear and spatial memories. Therefore in our experiment, accumulation of 4-7 week old DGCs at 8 wpi may be the underlying mechanism of the anterograde effect observed in this study.

4.5 p53 deletion degrade previously formed contextual fear memory

Previous studies focus mainly on the anterograde effect of neurogenesis changes, however manipulation of adult neurogenesis also affects memories encoded prior as reduced levels of neurogenesis is associated with better memory retention (Feng et al. 2001). Previous study in our group indicate that post-training voluntary running degrades previously acquired contextual fear and spatial memories causing retrograde amnesia (Akers et al. 2014). Similarly, the iKO-p53+/− mice that received TAM injections after CFC training exhibited significantly less freezing behavior when tested at 4 wpi indicating a degraded contextual fear memory.

However, in iKO-p53−/− mice subjected in the same experiment, a similar, yet not significant trend is observed. Since the immature DCX+ cells are increased more in iKO-p53−/− than in iKO-p53+/− mice, we initially hypothesized a more pronounced reduction of freezing behavior in iKO-p53−/− mice. More work has to be done to solve this problem, such as validating p53 deletion in iKO-p53−/− and iKO-p53+/− mice and subjecting them together in one experiment.

4.6 DCX+ DGCs do not represent functioning DGC population

In correlating the histological finding of increased neurogenesis to the behavioral outcome, it is worth noting that the DCX+ population does not directly represent functioning newborn cells. Instead, the expression of DCX precedes functional integration. Recombinant progenitor cells start to express DCX as early as 1 dpi of TAM (Lagace et al. 2007). Newborn neurons stop
expressing DCX before they fully mature, as scarcely any cells co-express DCX and Calbindin (CB), a marker for mature neurons in the SGZ (Spampanato 2012). In contrast, right before CB expression, the majority of Calretinin positive (CR+) cells in the SGZ co-express DCX (Jinno et al. 2011). The transition from CR to CB expression (and from DCX+ to DCX-) occurs at 3-4 weeks post-BrdU labeling (Brandt et al 2003). However as mentioned above, at 4 wpi immature neurons are anatomically connected but not functionally integrated into the local network (Ge et al. 2007). Therefore DCX+ cells are not necessarily integrated into hippocampal circuitry.

As such, if cell survival is enhanced during the transit from DCX+ to DCX- stage in iKO-p53-/- and iKO-p53+/- mice, the population of immature neurons participating in the hippocampal function could be increased to a greater degree than the DCX+ population. Alternatively, if the time window expressing DCX is shorten in the recombinant cells owing to development pattern altered by p53 deletion, the accumulated number of newborn DCGs would be higher than what is shown by DCX staining.

Hence to interpret these data, accumulation and long-term survival of functionally integrated immature DGCs should be examined. BrdU can be injected after TAM and at 4 wpi and 8 wpi of TAM the number of BrdU+ cells and BrdU+/CB+ can be assessed.

4.7 Future directions

4.7.1 Further characterization of adult neurogenesis

Due to time constraints, methodology and mice, characterization of neurogenesis in this study is not complete. Verification of the p53 deletion is an important next step. We would cross ROSA-reporter mice with iKO-p53-/- and iKO-p53+/- mice to produce double transgenic mice. As such Cre-ERT2-mediated recombination would be indicated by constitutive expression of a reporter gene.

Second, it would be important to quantify the magnitude of apoptosis blocked by p53 deletion, which can be assessed by Activated-Caspase-3 or TUNEL staining.

Third, to examine the effect of p53 deletion on the development and function of post-mitotic DGCs, we can construct a retrovirus-delivered ROSA-EGFP sequence and inject it prior to TAM injection. We can quantify the length and complexity of the dendrites of the recombinant cells.
and use slice recording to measure a series of electrophysiological parameters, such as LTP threshold, LTP amplitude and input resistance.

4.7.2 Establishing causal link between neurogenesis and behavior

In this study, we provide evidence that p53 deletion increases adult hippocampal neurogenesis and results in a series of behavioral changes. Although the result is in accordance with previous studies using other pro-neurogenesis models, the present study lacks a causal link between increased adult neurogenesis and behavioral changes.

To associate neurogenesis levels with behavioral performance, chemotherapy agents, such as TMZ and 5-fluoraracil, can be used to normalize neurogenesis in iKO-p53−/− and iKO-p53+/− mice to control levels. However chemotherapy agents have been found to cause brain-wide cytotoxicity (Deprez et al. 2012; Han et al. 2008; McDonald et al. 2010). A more specific method would be Cre-inducible diphtheria toxin receptor (iDTR) expression system (Buch et al. 2005; Imayoshi et al. 2008; Han et al. 2009). Arruda-Carvalho et al. (2011) have shown that, by crossing iDTR mice with Nestin-CreERT2 mice, iDTR was expressed specifically in neural progenitor cells and their progeny, and with DT injection, apoptosis was induced in these cells. Importantly, the specificity of this system has been demonstrated. First, DTR expression did not affect neurogenesis or induce apoptosis without DT. Second, DTR− murine cells were insensitive to DT (Arruda-Carvalho et al. 2011; Han et al. 2009).

We can deliver a ROSA-DTR sequence into iKO-p53−/− and iKO-p53+/− mice using viral or transgenic methods. By injecting a proper dose of DT, a proportion of recombinant cells would be ablated, normalizing the number of newborn cells in iKO-p53−/− and iKO-p53+/− mice to control levels. First, to verify that retrograde amnesia is caused by a post-training increase of synaptic remodeling and neurogenesis in the DG, DT should be injected following TAM injection and 4 weeks before the test. We predict that by normalizing neurogenesis, iKO-p53−/− and iKO-p53+/− mice should not display retrograde amnesia. Second, to verify that hippocampal learning is enhanced by increasing the number of immature neurons, TAM should be injected 8 weeks before behavioral training, as in this study, and DT should be injected directly before behavioral training. We hypothesize that by normalizing the number of immature neurons before the test, no learning facilitation should be observed in iKO-p53−/− and iKO-p53+/− mice.
4.7.3 Whether p53 deletion degrade previously acquired remote memory

On the basis of recent contextual fear memory being degraded by post-training p53 deletion, the next question is whether such a manipulation can degrade remote contextual fear memory as well as other remote hippocampus-dependent memories. Memory consolidation is the process by which labile short-term memories are stabilized to a more enduring state (Lechner et al. 1999; Frankland and Bontempi 2005). This process has two levels: synaptic consolidation, which stabilizes the synaptic connectivity of local memory circuits (such as the hippocampus which encodes spatial and contextual fear memories) within hours of training; and system consolidation, which involves slow and gradual reorganization of broadly distributed brain regions supporting memories (Frankland and Bontempi 2005). Classic consolidation theory proposes that declarative memories are initially stored in the medial temporal lobe (MTL) including the hippocampus. After system consolidation, memories are transferred to the cortex and become independent of the MTL (Squire 1992; Alvarez and Squire 1994). In patients and animal models, hippocampal lesion or disruption leads to loss of memories formed within a restricted period before the lesion, a phenomenon called temporally-graded retrograde amnesia (Rempel-Clower et al. 1996; Anagnostaras et al. 1999; Clark et al. 2002; Wang et al. 2009). Moreover, whether the hippocampus is necessary for the expression of remote memories may depend on the type of task. Previous studies uncover a temporally-graded retrograde amnesia in CFC (Anagnostaras et al. 1999; Wang et al. 2009) but a non-graded retrograde amnesia in MWM (Clark et al. 2005; Martin et al. 2005) after hippocampal lesions. To explore the effect of increasing adult neurogenesis on different types of previously acquired remote memories, we can induce p53 deletion at a remote time point (e.g. 5 week) after training in CFC or MWM and test them 4 weeks after the deletion.

However, different from those studies using acute lesion or disruption, increasing adult neurogenesis does not impact memory circuit directly. Rather it mimics natural forgetting through promoting gradual remodeling of local memory network as mentioned above. Therefore it is possible that during the remodeling, the altered hippocampal memory network may influence the cortical memory network, for example, weaken or degrade it.
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